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Disruption of the $G_{i2}\alpha$ locus in embryonic stem cells and mice: a modified hit and run strategy with detection by a PCR dependent on gap repair

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We have used an insertion vector-based approach to target the $G_{i2}\alpha$ gene in AB-1 embryonic stem cells. 105 bp located 0.8–0.9 kb upstream of a disrupting Neo marker in exon 3 were deleted and replaced with an engineered *Not* I site, that served to linearize the vector. The 105 bp deletion served as a primer annealing site in a polymerase chain reaction (PCR) designed to detect the gap repair associated with homologous recombination. Both target conversion and vector insertion events were obtained ('hit' step). Clones that had inserted the entire targeting vector were taken into FIAU (1-[2-deoxy, 2-fluoro-β-D-arabinofuranosyl]-5-ioduracil) counterselection to select against a thymidine kinase (TK) marker flanking the homologous genomic sequences and thus for cells that had excised the plasmid and the TK marker by intrachromosomal recombination ('run' step). Additional selection in G418 reduced the number of drug-resistant colonies at least five-fold. Thus, the Neo marker disrupting the homologous sequences allows for a more specific selection of the desired intrachromosomal recombination event in tissue culture. This modified 'hit and run' strategy represents a novel approach for vector design and the use of the polymerase chain reaction to detect targeting. It may be particularly useful for targeting genes that display a low frequency of homologous recombination. Germ line transmission of the mutated $G_{i2}\alpha$ allele is also demonstrated.

Keywords: Homologous recombination; gene targeting; intrachromosomal recombination; polymerase chain reaction; G protein

Introduction

The α subunits of G_i proteins (G_{i1}, G_{i2} and G_{i3}) are pertussis toxin substrates thought to be mediators of hormonal inhibition of adenylyl cyclase and stimulation of K^+ channels of the inwardly rectifying and ATP-sensitive types (reviewed in Birnbaumer, 1990, 1992; Birnbaumer *et al.*, 1990). While stimulation of K^+ channels has been shown in cell-free systems upon addition of GTPγS-activated α_i subunits (Yatani *et al.*, 1988; Kirsch *et al.*, 1990; Itoh *et al.*, 1991), inhibition of adenylyl cyclase has not been shown to occur in a cell-free system. Both effects are questioned in the literature. Activation of K^+ channels in response to α_i is proposed to be secondary to activation of phospholipase A_2 (Kurachi *et al.*, 1989; Kim *et al.*, 1989) and inhibition of adenylyl cyclase is proposed to be due to βγ dimers (reviewed in Gilman, 1984, 1987). Although experimental arguments were made that might indicate mediation of adenylyl cyclase inhibition by α_i subunits as opposed to βγ dimers (Wong *et al.*, 1991), their mediation of hormonal inhibition of adenylyl cyclase continues to be doubted (c.f. Tang and Gilman, 1992). The same situation surrounds the functionality of all three types of α_i.

As seen through the effects of pertussis toxin, G_i(s) have been implicated in addition in a variety of 'non-conventional' processes. These include the activation of phospholipase A_2 independent of phospholipase C activation (Burch *et al.*, 1986), the epidermal growth factor (EGF) induced activation of phospholipase C in liver cells

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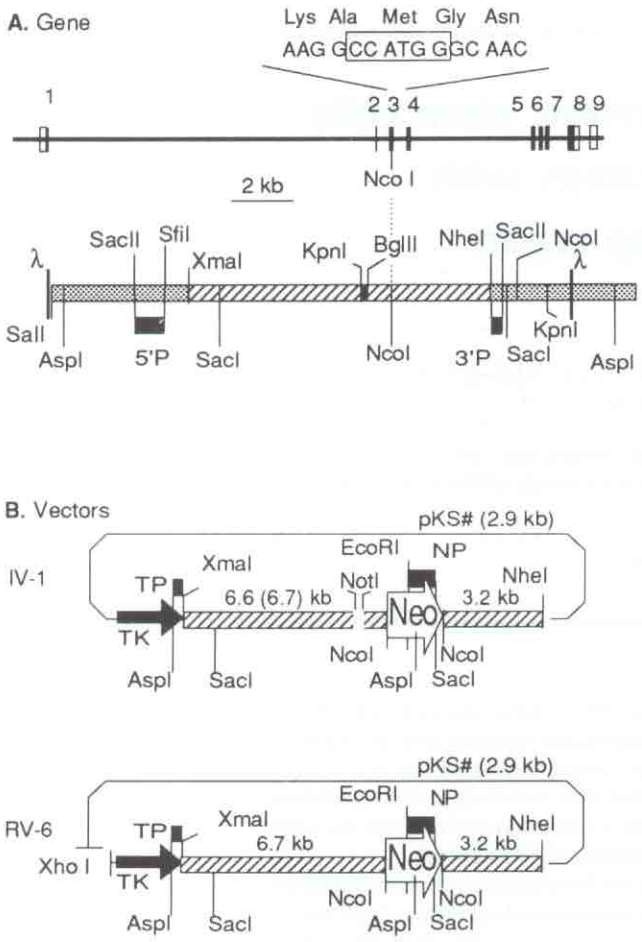

Fig. 1. Schematic representation of the structures of $G_{i2}\alpha$ gene, targeting vectors and expected recombinants at the $G_{i2}\alpha$ locus. A. Top: Intron-exon structure of the $G_{i2}\alpha$ gene and location of the *Nco* I site in exon 3. Below: Alignment of the insert of clone λEMBL3–α_{i2} along the intron-exon structure shown above with restriction sites relevant to this work. The thick vertical bars (λ) indicate the boundaries of the cloned gene. The hatched regions represent the genomic sequences that were included in the targeting vectors IV-1 and RV-6. The grey regions (dotted) indicate the genomic sequences that were not included in the vectors. The black boxes designated 5'P and 3'P indicate the 5' and 3' flanking probes, respectively, that were being used in Southern blot experiments (see Figs 3 and 5). The *Sal* I site was derived from the λ arm. B. Targeting vector IV-1 contains approximately 6.6 kb of homology 5' and 3.2 kb of homology 3' of the selectable Neo marker, which interrupts the $G_{i2}\alpha$ coding sequence in the third exon. Thus, an integration of this vector into the $G_{i2}\alpha$ locus is predicted to create a null allele. A TK cassette intended to act as a counterselection marker to select against clones that have inserted the entire targeting vector ('run' step, see Figs 4 and 5), is flanking the $G_{i2}\alpha$ genomic (hatched) sequences in IV-1. A 105 bp *Kpn* I–*Bgl* II fragment 0.8–0.9 kb upstream of the Neo marker which served as a primer annealing site in the PCR screen for homologous recombinants (Fig. 2) was deleted and replaced with a unique *Not* I site, which was used to linearize the plasmid. The positions of the DNA fragments used as internal probes in the Southern analysis (black boxes denoted *TP* (TK probe) and *NP* (Neo probe) are indicated. The arrows indicate the transcriptional orientation of the Neo and TK markers. Targeting vector RV-6 is identical to IV-1 except that the 105 bp *Kpn* I–*Bgl* II fragment was not removed. It was linearized outside of the homology with *Xho* I.

(Yang *et al.*, 1991), the colony stimulating factor I (CSF-I) and tumor necrosis factor (TNF) stimulation of proliferation of promacrophage cell lines (Imamura and Kufe, 1988; Imamura *et al.*, 1988), the activation of the acrosome reaction in capacitated sperm cells (Endo *et al.*, 1987), the negative regulation of vesicle budding from the trans-Golgi network (Leyte *et al.*, 1992), endosomal acidification (Gurich *et al.*, 1991), certain mitogen-induced stimulations of DNA synthesis such as lysophosphatidic acid (LPA)-mediated stimulation of thymidine incorporation into DNA of primary foreskin fibroblasts (van Corven *et al.*, 1989), de-differentiation of primary hepatocytes in culture (Itoh *et al.*, 1984), and mitogen-induced cell alkalinization (Paris and Pouysségur, 1986). The mechanism, be it direct or indirect, by which a G_i protein mediates these effects is unknown. In addition they may have unsuspected effects.

To gain a possibly novel insight into the function and mechanism of action of a $G_i\alpha$ subunit, we have set out to obtain a mouse strain with an inactivated $G_{i2}\alpha$ (α_{i2}) gene through homologous recombination in embryonic stem (ES) cells. The $G_{i2}\alpha$-subunit is expressed in all somatic cells studied so far, and also presumably in sperm. We report below the construction of two vectors, one an insertion and the other a replacement vector, with which this may be accomplished. Mortensen *et al.* (1991) have reported on the generation of ES cell clones with one or both $G_{i2}\alpha$ alleles targeted. However, the fate of these cells upon reintroduction into embryos has not been reported. We have obtained a cell line that gave rise to chimaeric mice carrying the mutation in the germ line.

Materials and methods

Insertion and replacement vectors

Insertion vector IV-1 contains a 6.7 kb *Xma* I–*Nco* I fragment upstream of the *Nco* I site in exon 3 of the $G_{i2}\alpha$ gene and a 3.2 kb *Nco* I–*Nhe* I fragment downstream of the *Nco* I site (Fig. 1A,B). The genomic fragments have been derived from BALB/c mice. The PolIINeobpA cassette (Soriano *et al.*, 1991) was inserted into this *Nco* I site. A 105 bp fragment between the *Bgl* II and *Kpn* I sites 0.8 kb and 0.9 kb upstream of the *Nco* I site in exon 3, was deleted and replaced by a linker consisting of a hybrid of oligonucleotides GT40 (5'–CAGCGGCCGCA–3') and GT41 (5'–GATCTGCGGCCGCTGGTAC–3') creating

a unique *Not* I site. The 5′ homology was flanked by a MCITK cassette. The plasmid backbone was pKS (Blue-Script, Stratagene).

Replacement vector RV-6 is identical to insertion vector IV–1 except that the 105 bp *Kpn* I-*Bgl* II fragment was not removed (Fig. 1B).

Electroporations and tissue culture

General procedures described in Sambrook *et al.* (1989) were used for manipulation of DNAs. The techniques for manipulation of ES cells were those described by Robertson (1987). The DNA for electroporations was prepared by alkaline lysis and banded once in CsCl. Linearization was followed by phenol/chloroform and chloroform/isoamyl alcohol extraction and analytical gel electrophoresis. The insertion vector IV–1 was cut at the *Not* I site created in the gap described above. The replacement vector RV-6 was linearized at a *Xho* I site outside the region of homology between the MCITK cassette and the plasmid backbone. Electroporations were performed using AB-1 ES cells (McMahon and Bradley, 1990) at passage number 13 and 20 in Experiments 1 and 2, respectively (see Table 2). Cells were resuspended in phosphate-buffered saline at a density of 10^7 cells per 900 μl, and then 25 μl of DNA was added at a concentration of 1 μg μl^{-1}. The Bio-Rad Gene Pulser was used with 0.4 cm cuvettes at a setting of 230 V and 500 μF. After the electrical shock, the cells were kept for 5–10 min at room temperature before plating at a density of 125000–250000 cells per cm^2 onto a layer of mitomycin C-treated STO feeder cells in 100 mm-diameter plates or multi-well plates. The plating efficiency was approximately 70%. The culture medium contained 10–15% Defined Fetal Bovine Serum (Hyclone), 10^{-4} M 2-mercaptoethanol (Sigma), 2 mM glutamine, penicillin 100 units ml^{-1}, streptomycin (100 μg ml^{-1}) (all from Gibco) and supernatant (200 μl l^{-1} of the LIF-producing CHO cell line 8/24 720 LIF-D(.1) (Genetics Institute). Selection with G418 (180 μg ml^{-1} active ingredient, Gibco) or G418 plus FIAU (0.2 μM; Bristol–Myers) were initiated 1 or 2 days post electroporation. After 9–14 days, colonies were picked and expanded. Colonies derived from IV-1-transfected ES cells were trypsinized in a 15 μl trypsin spot and disaggregated with 20 μl of fresh medium. To 25 μl of PCR (polymerase chain reaction) lysis buffer (1 × Cetus PCR buffer containing 0.45% NP40, 0.45% Tween 20 and 400 μg ml^{-1} Proteinase K), 15 μl of the cell lysate was added and incubated at 60° C overnight. Of this lysate, 2 μl was used in a 20 μl PCR (see below).

For preparation of genomic DNA, cells were lysed in a buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM Na-EDTA, pH 8.0 and 0.5% SDS and the lysates treated with 800 μg ml^{-1} proteinase K at 60° C overnight, followed by extraction with phenol, phenol/chloroform and chloroform/isoamyl alcohol, and precipitation with isopropanol. The DNA was either spooled on a flame-sealed micropipette or harvested by a 5 min centrifugation at 10000 × **g**, washed twice with 70% ethanol and allowed to air-dry before resuspension in TE (10 mM Tris-HCl, pH 8.0, 1 mM Na-EDTA).

Polymerase chain reaction

PCR was performed for screening clones obtained with the insertion vector IV-1 using GT38 (5′-TCCAGCCAG-CCCTGCCTTGCTCTTT-3′) as an upstream primer hybridizing to sequences in the repaired gap described previously and thus not contained in the transfected targeting vector and GT36 as downstream primer that hybridizes to sequences close to the 5′ end of the PolIINeobpA cassette (5′-TTTACGGAGGCCTGGC-GCTCGATGT-3′). The primers were used at a concentration of 0.5 μM, the dNTP concentration was 200 μM. Two μl of cell lysate (see above) were added per 20 μl reaction. One unit of *Taq* DNA polymerase was used per 20 μl reaction with the buffer supplied by the manufacturer (Promega). The cycling profile using the Perkin Elmer Cetus DNA Thermal Cycler was 4 min initial melting at 94° C, then 50 cycles of 1 min at 94° C, 1 min at 65° C and 2 min at 72° C. Using the Ericomp Twin-Block System, the cycling profile was 3 min initial melting at 94° C followed by 50 cycles of 20 s at 94° C, 20 s at 65° C and 2 min at 72° C. The reaction products were then subjected to gel electrophoresis and transferred to GeneScreenPlus membranes (NEN). The identity of the amplified fragments was confirmed by Southern blotting with oligonucleotide GT48 (5′-CAGACAC-ACCAGAAGAGGGCATCA-3′), which hybridizes to sequences in the aforementioned gap downstream of oligonucleotide GT38.

Reversion of clones that had integrated insertion vector IV-1 by single reciprocal recombination

Cells were plated at a density of approximately 12500/cm^2 onto 10 cm plates containing STO feeder layers. FIAU (0.2 μM), or FIAU (0.2 μM) plus G418 (180 μg ml^{-1} active ingredient), were added at the time of plating (day 0) or at days 1, 2 or 3. The reversion rates were calculated according to Hasty *et al.* (1991a), by averaging the reversion frequencies between days 1–2 and days 2–3 using the equation: reversion rate = (number of colonies on day x − number of colonies on day $x-1$)/total number of cells on day x.

Southern analysis of targeted clones

Genomic DNA (5–10 μg) was digested with the enzymes indicated and fragments separated on a 0.7% agarose gel in 1 × TAE (40 mM Tris-acetate, 1 mM Na-EDTA) buffer. DNA was transferred onto GeneScreenPlus membranes (NEN) and hybridized to the probes indicated. *Asp* I-digested DNA was hybridized to a 5′-flanking

probe containing intron sequences between the *Sac* II site 8.4 kb upstream of the *Nco* I site of exon 3 and the *Sfi* I-site 7.6 kb upstream of the *Nco* I site or a *Sac* II–*Sma* I probe (8.4–6.7 kb upstream of the *Nco* I site), to a Neo probe, obtained by cutting the PolIINeobpA cassette with *Eco* RI and *Xba* I and a *Nco* I–*Cla* I TK probe including nucleotides 2023–2249 of the *hs1tk3kb* (i.e. HSV1TK) sequence (GenBank). *Sac* I-digested DNA was hybridized against a *Nhe* I-*Sac* I 3′ flanking probe 3.2–3.5 kb downstream of the *Nco* I site of exon 3 or a *Nhe* I-*Sac* II probe 3.2–3.8 kb downstream of the *Nco* I site in exon 3. The labelled copy DNA probes were made by random priming to specific activities of ca. 0.5–1.5 × 10^9cpm μg^{-1} according to Feinberg and Vogelstein (1983). The last wash of the membranes was usually performed at 65° C using 0.2 × SSC, 0.5% SDS. Kodak XAR5 films were exposed between 1 to 14 days at −70° C using 2 intensifying screens.

Generation and analysis of chimaeric mice

Manipulations were carried out as described (Bradley, 1987). In short, C57BL/6J blastocysts were isolated at day 3.5, injected with 10–15 stem cells (clones 31A, 67D and 75B, passage numbers 23, 22 and 22, respectively) and reimplanted into 2.5 day pseudopregnant C57BL × CBA foster mothers. Clone 67D is a subclone of clone 67, clone 31A a subclone of 31, which was identical to 67 in Southern analysis, and clone 75B is a clone derived from 75, identical by Southern analysis to 75R (see below). Genomic DNA prepared from tail biopsies was analysed by blot hybridization. Male chimaeras were bred to C57BL/6J females and the genotype of the offspring was identified as described above.

Results

In order to target the murine $G_{i2}\alpha$ gene, an insertion vector, IV-1 (Fig. 1B), was constructed containing 6.6 kb and 3.2 kb of homologous sequences separated by the PolIINeobpA cassette (Soriano *et al.*, 1991) inserted in the *Nco* I site of exon 3. A 105 bp fragment 0.8–0.9 kb upstream of the Neo cassette was deleted and replaced with an engineered *Not* I restriction site. An MCITK cassette was placed adjacent to the 5′ homologous sequences. We predicted that the integration of this vector into the target locus would repair the gap (Jasin and Berg, 1988; Valancius and Smithies, 1991; Hasty *et al.*, 1992) and create a null allele. To detect homologous recombinant stem cell clones, a fragment specific for the homologous recombinant was amplified by a PCR (Fig. 2). The upstream primer, GT38 or 'Gap' primer, hybridized to sequences of the 105 bp fragment deleted in IV-1; the downstream primer, GT36 or 'Neo' primer, hybridized to the 5′ end of the PolIINeobpA cassette. The PCR products were hybridized against the external probe GT48 to confirm their identity. Out of 96 colonies, 16 scored positive. Twelve clones were subjected to genomic Southern analysis to confirm that homologous recombination had occurred (Fig. 3). DNA was digested with *Asp* or *Sac* I and probed with 5′ and 3′ flanking, Neo and TK probes. In this analysis, 10 out of 12 clones analysed were confirmed to be homologous recombinants. The other two clones might have resulted from vector conversion by the target locus (Adair *et al.*, 1989; Pennington and Wilson, 1991). Five clones had the targeting vector inserted by single reciprocal recombination resulting in a duplication of homology. Three of them (74, 83 and 86) had the Neo cassette in the 5′ duplicate. Two clones (75 and 77) had the Neo marker in the 3′ duplicate, indicating branch migrations of the Holliday junctions past the Neo cassette and resolution on the 3.2 kb arm of the vector. Three clones (29, 31 and 67) had the structure predicted for a target conversion event (Adair *et al.*, 1989; Pennington

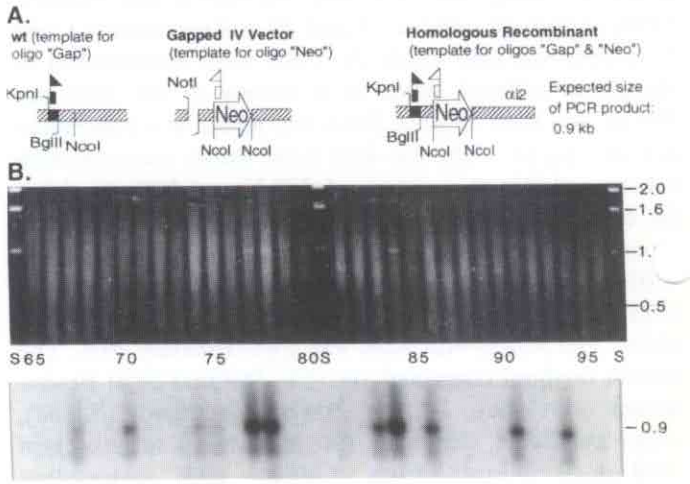

Fig. 2. PCR amplification of a fragment specific for the IV-1 targeted $G_{i2}\alpha$ allele. A: Schematic representation of the PCR strategy. Homologous recombination is predicted to repair the gap present in the targeting vector, thus creating an annealing site for oligonucleotide GT38, denoted as 'Gap' on the figure, which will then together with oligonucleotide GT39, denoted 'Neo' on the figure, amplify a 0.9 kb junction fragment specific for the recombinant allele. The flag symbols represent the primer annealing sites. B: Analysis of stem cell clones after electrophoretic separation of PCR products obtained from clones 65–96. Colonies were lysed and PCR amplifications performed as described in Materials and methods. Top: Photograph of ethidium bromide-stained 1% agarose gel. Bottom: Hybridization of the PCR products shown in the top panel after transfer to a GeneScreenPlus membrane (NEN) to external oligonucleotide probe GT48. The final wash was at 65° C in 0.2 × SSC/0.5% SDS. The numbers between the top and bottom panels identify separate lanes and refer to the clone designations used throughout in this report (see also Fig. 3). 1-kb DNA ladder (BRL) used as a sizing standard.

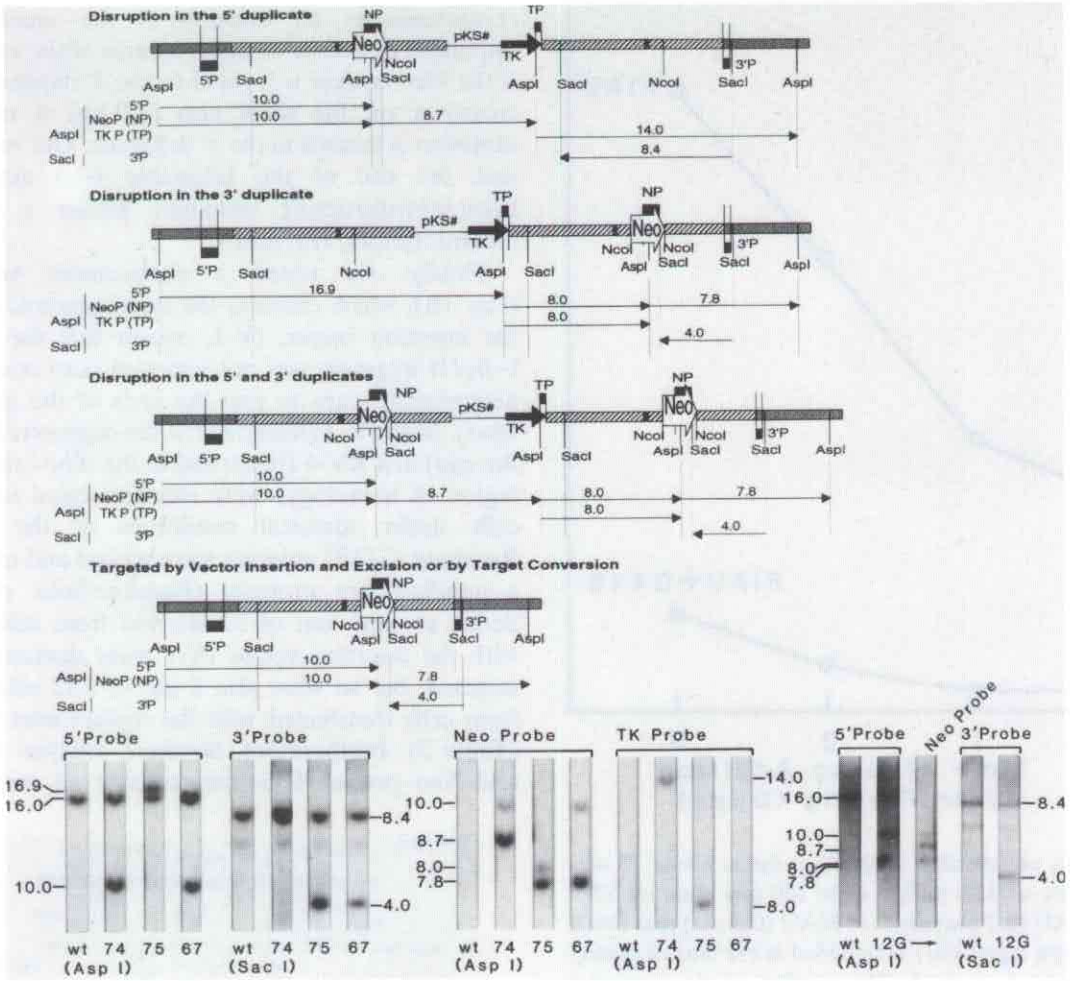

Fig. 3. Southern analysis of restriction digests of DNA from IV-1 targeted ES cell clones with external 5′-flanking, external 3′-flanking, Neo and TK probes. DNA samples from untransfected ES cells and PCR-positive cell lines were digested with the indicated restriction enzymes and hybridized against the indicated probes. Clone 67 did not hybridize with TK or pKS sequences (not shown), indicating that those sequences have not been incorporated into the target locus and this clone has undergone target conversion. Clone 74 had the Neo marker in the 5′ duplicate, clone 75 in the 3′ duplicate and clone 12G in both duplicates. Hatched bars, $G_{i2}\alpha$ sequences present in the vector; black bars, 5′ and 3′ flanking sequences of the locus from which 5′ and 3′ flanking probes, black boxes denoted 5′P and 3′P, were derived.

and Wilson, 1991). Since IV-1 offered homologous free ends, it is likely that they were used in the recombination process (Hasty *et al.*, 1992). These clones probably arose by branch migration of a Holliday junction, heteroduplex mismatch repair to the Neo sequences and resolution of the Holliday junctions in a non-crossover plane. In a separate experiment, 2 clones (12G and 6E) were recovered that had the Neo marker in both duplicates. This can be explained with repair synthesis to the Neo marker of two sets of heteroduplexes (Hasty *et al.*, 1992). Two clones (19 and 53) were targeted with an unexplained integration pattern (not shown).

In order to determine whether a duplication of genomic sequences with one duplicate containing a 1.8 kb heterology (i.e. the Neo cassette) not present in the other duplicate can undergo intrachromosomal recombination ('run' step) we determined the reversion frequency for clone 75, which had the Neo marker in the 3′ duplicate, by testing for loss of TK activity by selection with FIAU alone or FIAU plus G418 (Fig. 4). The former condition should select for all reversion events (excision to the mutant genotype with the Neo marker present, excision to the wild-type, spontaneous loss of TK function), while the latter condition should select specifically for an excision to the mutant genotype plus spontaneous loss of TK function without spontaneous loss of Neo function. The G418 selection should also eliminate chromosome loss events from the background. The total reversion frequency

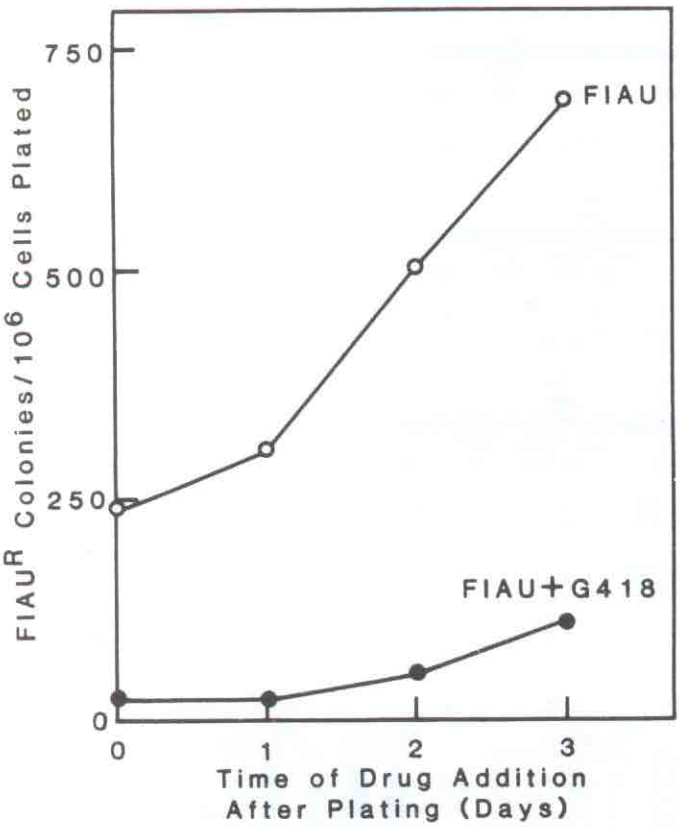

Fig. 4. Frequency of reversion at the $G_{i2}\alpha$ locus. Clone 75 was plated at a density of 0.96 million cells/100 mm plate on STO feeder layers. FIAU (0.2 μM) alone or FIAU (0.2 μM) plus G418 (180 μg ml^{-1} active ingredient) were added at the time of plating (day 0) or at days 1, 2 or 3. The number of drug-resistant colonies is plotted as a function of the time of drug addition. The results indicate that an additional selection with G418 dramatically decreases the background of colonies that have excised the duplicate containing the Neo marker or that have spontaneously lost the Neo function.

(selection with FIAU alone) was estimated to be 4.6 × 10^{-5} per cell generation. Most of these clones did not have a functional Neo marker since they were killed by the addition of G418 to the medium. The 'mutant-specific' reversion frequency (as detected by selection with FIAU and G418) was estimated to be 8.1 × 10^{-6}. Southern analysis (Fig. 5 and summarized in Table 1) showed that six out of seven G418^{R}FIAU^R colonies obtained after plating clones 75 and 77 had the structure of the predicted excision event to the mutant genotype (shown for clone 75 in Fig. 5), with the remaining clone being identical to the parent clone, possibly indicating a mutation disabling the TK gene. We also obtained mutant excision revertants from clone 74, which had the Neo marker in the 5′ duplicate, although at a relative frequency that was one order of magnitude lower than that obtained with clone 75 (Table 1). This difference can be explained by the length of homology that is available for intrachromosomal recombination; an excision to the mutant genotype requires a crossover on the long arm of the vector (6.7 kb) if the Neo marker is located in the 3′ duplicate whereas a crossover on the short arm (3.2 kb) is needed if the mutation is located in the 5′ duplicate. Our results indicate that the use of the selectable Neo marker as the homology-disrupting mutation allows a selection of mutant excision revertants.

Finally, we tested a replacement vector, RV-6 (Fig. 1B), which contains the same genomic sequences as the insertion vector, IV-1, except that the 105 bp *Kpn* I–*Bgl* II fragment was not removed (and consequently no heterologous caps to join the ends of the gap had to be used). Both IV-1 (linearized at the engineered *Not* I site in the gap) and RV-6 (linearized at the *Xho* I site outside the region of homology) were electroporated into AB-1 ES cells under identical conditions at the same time. Resultant G418^R colonies were picked and analysed using a miniSouthern protocol (Ramírez-Solis *et al.*, 1992). Seven colonies out of 92 derived from cells transfected with the insertion vector IV-1 were demonstrated to be targeted, but so were also 8 out of 132 colonies derived from cells transfected with the replacement vector RV-6 (Table 2). Furthermore, Southern analysis with flanking and Neo probes demonstrated that all targeting events

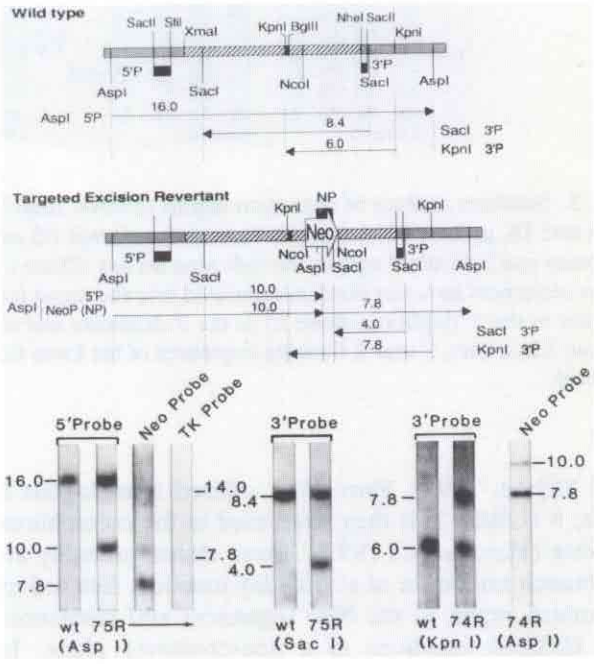

Fig. 5. Southern analysis of revertant G418^{R}FIAU^R clones. DNA samples from untransfected ES cells and revertant G418^{R}FIAU^R subclones 74R and 75R derived from clones 74 (Neo marker in the 5′ duplicate) and 75 (Neo marker in the 3′ duplicate), respectively, were digested with the indicated restriction enzymes and hybridized against the indicated probes. Both clones had lost the duplication but have retained the N marker, thus demonstrating excision of the wild-type duplicate.

Table 1. Summary of intrachromosomal recombinations at the $G_{i2}\alpha$ locus

Experiment	*Location of the Neo marker*	*Clone tested for reversion*	*Homology available for recombination*	*Relative frequency of excision after FIAU and G418 selection*
A	3′ duplicate	75	6.7 kb	3/4
		77		3/3
				Sum: 6/7
	5′ duplicate	74	3.2 kb	0/4
		83		0/4
		86		0/3
				Sum: 0/11
B	5′ duplicate	74	3.2 kb	6/53

Clones that had been targeted by vector insertion were plated onto feeder cells at a density of 10^5 cells per 10 cm plate (Experiment A) or 10^6 cells per 15 cm plate (Experiment B). Two days later the cells were subjected to FIAU plus G418 selection as described in Materials and methods. Colonies were picked 9–14 days later and expanded. Their DNA was analysed by Southern blotting for loss of the duplicate without the Neo marker as shown in Fig. 5.

obtained with the replacement vector RV-6 had the predicted target structure; one of these clones had also integrated the targeting vector at a random site (not shown). The types of targeting obtained in experiments 1 and 2 are summarized in Table 3.

Three clones, 31A, 67D and 75B were injected into C57BL/6J blastocysts, which were reimplanted into pseudopregnant foster mothers (see Materials and methods). Two of them, 31A and 67D, gave rise to chimaeric mice. As judged by the agouti coat colour marker, the chimaeras derived from clone 67D had a degree of chimaerism of approximately 30%, those derived from clone 31A of approximately 90%. Southern blots of tail biopsies demonstrated the presence of the mutated allele in all chimaeras derived from clone 31A (Fig. 6). The six chimaeras derived from clone 31A which have been tested were shown to transmit the mutated $G_{i2}\alpha$ allele in the germ line (see also Fig. 7).

Table 2. Summary of targeting frequencies obtained with IV-I and RV-6

Experiment	*Vector*[a]	*$G418^R$ Resistant cells per 10^7 cells plated*	*Targeted $G418^R$ clones*
1	IV-1	1837	10/72
2	IV-1	1624	7/92
	RV-6	810	8/132

[a] For comparison of structures see Fig. 1.

Discussion

The targeting frequencies for different genes may vary by three orders of magnitude and cannot be predicted (Camerini-Otero and Kucherlapati, 1990). They have been shown to be influenced by vector design and the length and degree of homology (Thomas and Capecchi, 1987; Hasty *et al.*, 1991b, 1991c; Deng and Capecchi, 1992; te Riele *et al.*. 1992). The vector design most commonly used is the replacement vector with a selectable Neo marker disrupting the homology enabling a 'positive' selection with G418. By placing one or two TK markers outside of the homology, against which one can select with gancyclovir or FIAU, a partial 'negative' selection against random integration events is possible; enrichments of homologous recombinants of up to 2000-fold (Mansour *et al.*, 1988; Johnson *et al.*, 1989) have been described. However, marginal 2-fold enrichments have also been reported using this selection procedure (Zijlstra *et al.*, 1989; Mombaerts *et al.*, 1991). An alternative type of vector design is the insertion vector, which is defined by a double-strand break within the region of homology. Whereas a replacement vector is integrated into the target locus by double reciprocal recombination or gene conversion, an insertion vector takes part in a single reciprocal recombination which can result in the targeted insertion of the whole targeting vector, target conversion (replacement of a genomic sequence) or vector conversion (not

Table 3. Targeting patterns obtained with IV-1 and RV-6 at the $G_{i2}\alpha$ locus

Genomic structure and location of disruption	*Targeting vector*[a]	
	Insertion (IV-1)	*Replacement (RV-6)*
Total no. of targeting events	17	7
Disruption in 5′ duplicate	6	–
Disruption in 3′ duplicate	2	–
Disruption in both duplicates	2	–
Replacement-type event	4	7
Unexplained	3	–

[a]For comparison of structures see Fig. 1. The data shown for IV-1 represent the combined results of Experiments 1 and 2 (see Table 2).
Note that the replacement-type events obtained with IV-1 most likely involve the free ends of the targeting vector and are thus mechanistically different from those obtained with RV-6 (double crossover or gene conversion).

disrupting the target locus) (Adair *et al.*, 1989; Pennington and Wilson, 1991). There is currently no general agreement on whether insertion vectors target at the same (Thomas and Capecchi, 1987; Deng and Capecchi, 1992) or a higher (Jasin and Berg, 1988; Hasty *et al.*, 1991b) frequency than replacement vectors. We report here similar targeting frequencies for a replacement vector (RV-6) and an insertion vector (IV-1) containing essentially the same homologous sequences. Experiments not reported here (P. Hasty and A. Bradley unpublished results) showed that the targeting frequency of insertion vectors is decreased 2.5-fold by the presence of a 500 bp gap. Likewise, the break in homology due to the introduction of a heterology, here the Neo marker, also decreased the targeting frequency, by 2-fold, independent of the size of heterology. Short heterologous caps on the free ends (here the *Not* I restriction site) may also reduce the targeting frequency by approximately 1.6-fold (Hasty *et al.*, 1992). Thus, the targeting frequency of a putative IV-1-like insertion vector without a gap (and linearized at a naturally occurring restriction site) might be expected to be somewhat higher than that of RV-6.

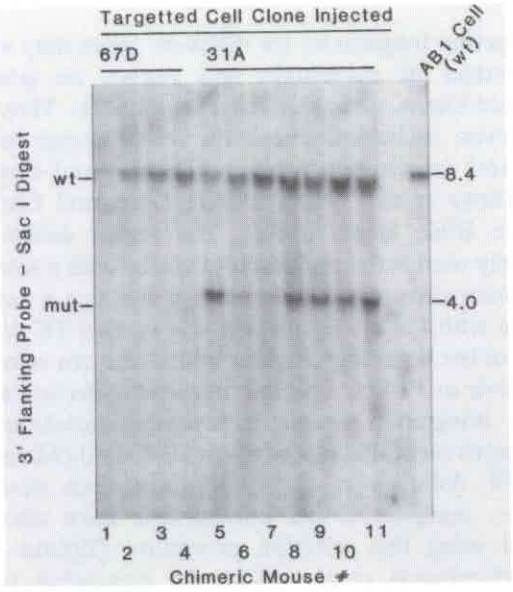

Fig. 6. Genotype of tail biopsies from chimaeric mice. Genomic DNA was prepared from tail biopsies of chimaeric mice that have been obtained through blastocyst injection, cut with *Sac* I, electrophoresed, transferred to a GeneScreenPlus membrane (NEN) and hybridized against a 3′ flanking probe. The mutated allele which creates a 4.0 kb *Sac* I–*Sac* I fragment could be detected in the biopsies from all chimaeric animals derived from clone 31A, but not in those derived from clone 67D.

A popular technique to screen for gene targeting events has been the PCR, which has quite frequently been used in conjunction with replacement vectors (Kim and Smithies, 1988; McMahon and Bradley, 1990). To develop a polymerase chain reaction, one arm of the replacement vector has to be sufficiently short to allow amplification with Neo and external primers. However, Thomas *et al.* (1992) reported recently that if one arm of the vector is reduced to below 1 kb in length, the fidelity of homologous recombination is diminished. This is in agreement with our own experience that a replacement vector with a 0.9 kb short arm was unable to target the $G_{i2}\alpha$ gene (not shown). We found that our PCR screen that detects the gap repair was able to detect one recombinant clone in a pool of at least 12 clones (not shown). Indeed, in our initial screen we had made pools of 8 or 12 clones, and found most of them to score positive (not shown). Had the recombination frequency at the $G_{i2}\alpha$ locus been very low, the PCR screen might have been valuable in identifying target clones.

Using an approach based on an insertion vector, a 'F' and Run' strategy has been developed to introduce sub...

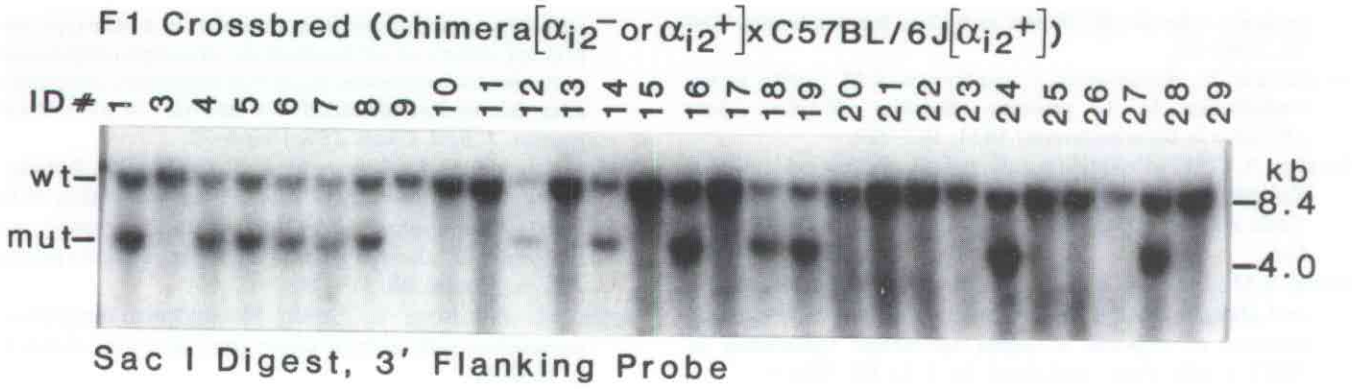

Fig. 7. Genotype of tail biopsies from F_1 mice resulting from a cross between male chimaeras and C57BL/6J females. Southern blot hybridization was performed as described in the legend to Fig. 6. Mice numbered 1–13 are descendants of chimaera 5, mice 14–16 of chimaera 6, 17–19 of chimaera 8, 20–27 of chimaera 9, and 28 and 29 of chimaera 11. The presence of the mutated allele in F_1 mice demonstrates that the chimaeric mice carry the mutation in the germ line.

mutations into the mammalian genome (Hasty *et al.*, 1991a). Here, we describe modifications of this 'Hit and Run' procedure which make it possible: (1) to use an insertion-based approach that creates mutant alleles that are identical to those obtained with replacement vectors, i.e. with a selectable marker disrupting the targeted gene and finally without any duplication of genomic sequences; (2) to use a PCR screen that is dependent on the gap repair that accompanies homologous recombination; and (3) to select directly for the desired excision event through the inclusion of the selectable marker in the mutated allele. This enables a three-fold selection. First, selection with G418 for clones that have integrated the Neo marker; then, after identification of the targeted events, with FIAU for clones that have excised one of the duplicates, the plasmid and the TK marker; and, at the same time, with G418 for those clones that have retained the duplicate with the mutation and excised the duplicate with the wild-type structure. It should also be noted that 30% of IV-1-targeted clones obtained in Experiment 1 (i.e. 4% of all recombinant events analysed) had undergone target conversion and therefore contained no duplication of genomic sequences.

The data presented in this paper demonstrate that the $G_{i2}\alpha$ locus can be targeted with both insertion and replacement vectors at high frequencies. Targeting with a replacement vector with two long arms of homology like RV-6 displays a high fidelity of homologous recombination but practically precludes a PCR screen to detect homologous recombinants. Screening therefore has to be done using Southern blot analysis. By using a counter-selection against the TK marker in RV-6 one could probably enrich for homologous recombinants. However, using a different replacement vector with two TK markers flanking the homology, we obtained only a 4.9-fold apparent enrichment at the $G_{i2}\alpha$ locus (not shown). The modified hit and run strategy described in this paper is insertion vector-based and thus utilizes a different mechanism for homologous recombination, which may provide a chance for success when other strategies do not work. The potential disadvantages of the modified hit and run scheme are that vector construction may be more complicated and time-consuming and with two selection schemes more cell manipulation is required, which can lead to decreased germ-line colonization efficiency. The latter disadvantage is partially offset by the relatively high frequency of gene conversions (see above) which may make a 'run' step unnecessary. We suggest that our approach may be useful in cases where conventional strategies have failed, particularly if the gene of interest apparently has a low targeting frequency.

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